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ON THE EFFECT OF AERATION AND NUTRITION ON CELLULOSE DECOMPOSITION BY CERTAIN BACTERIA

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Microbiological decomposition of cellulosic fibers, particularly cotton, is one of the major problems confronting the Quartermaster Corps in the South Pacific. Since the nature of the organisms concerned is of primary consideration, isolations were made from 150 samples of fabrics received from the tropics, in different stages of decomposition. Of these, the bacterial isolates were studied by the writers. Of nearly 500 such isolates, 39 or roughly 8 per cent were capable of destroying cellulose. These can be divided readily into two groups: (a) a highly aerobic type, chiefly *Cytophaga* sp., and (b) a type requiring little, if any, oxygen. The latter is not truly anaerobic as it is capable of growing in the presence of oxygen, although it does not respond to differences in the oxygen concentration. In order to explore the physiology of these two groups and to determine differences in their activity on cellulose, a study of one representative isolate from each group was undertaken. In this way, it was thought that it would be possible to provide conveniently more detailed information than could be obtained from a generalized examination of many or all of the isolates. As representative of group (a) was chosen an isolate of *Sporocytophaga myxococcoides*, and of group (b) *Cellulomonas* sp.

No method capable of giving satisfactory quantitative data regarding the rate of cellulose decomposition by aerobic organisms has heretofore been reported. To be effective, a procedure must be simple enough to permit determinations on a large number of samples in a relatively short time so that many variables can be studied simultaneously. The results must be reproducible and the replicates in such close agreement that a small number will yield significant results. Finally, the method must be able to demonstrate a selective response by the organism to small changes in the environment. As will be shown in subsequent pages, a method fulfilling these conditions was found in the use of shake flasks.

PROCEDURE

The difficulties involved in a study of cellulose decomposition by aerobic microorganisms are caused primarily by the fact that cellulose is insoluble in water. A satisfactory substitution for solution can be obtained, however, by mechanically or chemically reducing the cellulose to the finest particles that still possess the properties of the original material.

Approximately 4 g of ground filter paper was placed in a Waring "blendor,"

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and the desired mineral salts solution was added. After 2 minutes of agitation the suspension was transferred to a flask and made up to 1,000 ml with the same solution. Twenty-five-ml aliquots were placed in 250-ml Erlenmeyer flasks, which were plugged with cotton and sterilized. Substances to be tested were then introduced in sterile 1-ml portions, after which the flasks were inoculated with 0.25 to 0.50 ml of a suspension of the organism. The accompanying uninoculated control flasks were used for determinations of the original amount of cellulose and for the original determinations of hydrogen ion concentration.

The mineral solution selected was that of Fuller (1942) containing 0.1 per cent $NaNO_3$, 0.1 per cent K_2HPO_4, 0.05 per cent $MgSO_4 \cdot 7H_2O$, 0.05 per cent KCl, 0.003 per cent yeast extract Difco (in place of yeast water), and 0.4 per cent ground filter paper. Twenty-five ml of the suspension per flask was found convenient.

After a period predetermined for each organism, the flasks were removed from the shaker. The pH of the contents was determined by means of a Beckman pH meter. Two and one-half ml of N/1 KOH was then added to the contents, and the flask was autoclaved for 15 minutes at 15 pounds' pressure. The residue was filtered while hot through a Gooch crucible, washed with water, and dried at 102 C for 16 hours. After cooling, the crucibles were weighed, ignited in a muffle furnace, and reweighed. The uninoculated controls therefore represent the original weight of *ash-free* cellulose, and the differences (control minus test) are losses in organic matter. Since no correction was made for the weight of the bacteria, the actual percentage of loss in *cellulose* was always greater than that reported.

Filtration. One of the first problems in technique to be solved was that of filtering. It was soon observed that at least 72 hours was required to filter the 25 ml of residual mixture in the flasks after incubation. In order to speed up this operation several methods were tried, the KOH heat treatment described above proving most satisfactory By this method, the time required for filtering was reduced to 5 to 15 minutes, although an occasional sample required up to 30 minutes. The effect of the 0.1 N KOH and autoclaving on various substrates is shown in table 1.

The results reveal only slight decomposition of the original cellulosic substrates, except for the cellulose dextrin with which the KOH treatments result in a partial dissolution. The effect of alkali on the residue is much greater than on the pure substrate. It is believed that this is a result of the decomposition of bacteria and bacterial mucilage by the treatment, although the possibility exists that intermediates of cellulose, similar to the dextrins described above, are removed. In either case, it is thought that such treatment, in addition to speeding up the filtering, gives a closer approximation to the actual amount of cellulose decomposed.

Fuller (1942) also used an alkali treatment, but without heat, and followed by 1 per cent acetic acid. His purpose was not, however, related to the problem of filtration described above.

TABLE 1

Effect of prefiltering treatment with KOH on loss in weight of cellulose and of cellulose residues

SUBSTRATE	% LOSS IN WEIGHT DUE TO KOH + AUTOCLAVING
Cellulosic material	
Filter paper, ground	0
Cotton fabric	3
Cellulose dextrin (from H_2SO_4)	78*
Residues: filter paper—	
7-day decomposition by *S. myxococcoides*	10
11-day decomposition by *S. myxococcoides*	21
7-day decomposition by *Cellulomonas* sp.	8
7-day decomposition by *Spirochaeta cytophaga*, Gray's USDA	11
11-day decomposition by *Spirochaeta cytophaga*, Gray's USDA	16

* Without KOH there was a 38 per cent loss on autoclaving.

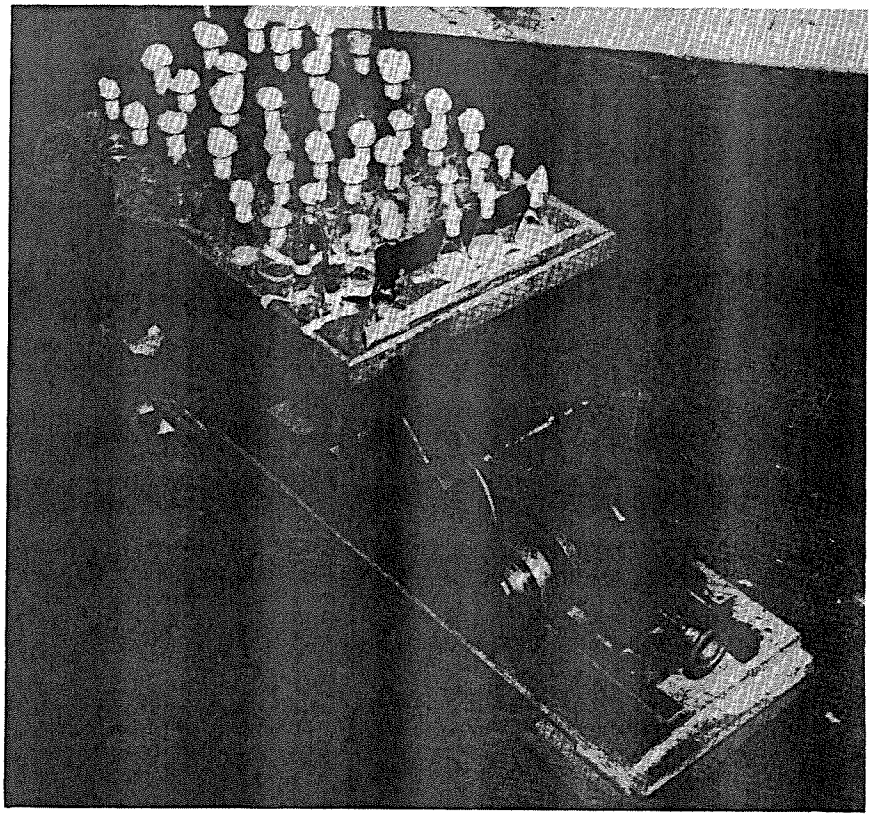

FIG. 1. SHAKER MODIFIED TO GIVE 120 CYCLES PER MINUTE

A definite contribution to the problem of aerobic decomposition of cellulose was made by Fuller and Norman (1943) when they bubbled air through large volumes of a filter paper suspension in order to obtain quantities of residue for chemical analysis. Since our objective was to devise a method for studying the optimal conditions for decomposition, it was possible to decrease the volume of suspension to 25 ml, aerated in 30-ml micro-Kjeldahl flasks. Several experiments employing this technique proved the method to be entirely adequate, but difficulties of manipulation (foaming, evaporation, sterility, and aeration rate) made the method less desirable than the shaking technique subsequently used.

TABLE 2

Effect of flask size on decomposition of filter paper on a shaker doing 120 cycles per minute

ORGANISM	% LOSS IN WEIGHT*		
	Shaken flasks		Unshaken
	125 ml	250 ml	250-ml flask
Sporocytophaga myxococcoides	54	54	13
Cellulomonas sp.	65	58	32

* Seven-day incubation period; 25 ml of medium.

TABLE 3

Effect of flask size on decomposition of filter paper by Cellulomonas sp.

FLASK SIZE	SUSPENSION	DEPTH	AVERAGE % LOSS IN WEIGHT*	
			Open flasks†	Sealed flasks‡
ml	*ml*	*mm*		
250	25	5	14	—
125	25	9	19	19
125	50	16	24	21
50	25	15	34	27

* Six-day incubation period.
† Cotton stoppers.
‡ Sealed with paraffin.

AGITATION BY SHAKE FLASKS

The speed of the reciprocal motion box shakers commonly available is much too high for use in bacteriological work. Reduction of the speed to 120 complete strokes per minute has been found satisfactory with 125-ml and 250-ml flasks.

Since 100 mg of ground filter paper in 25 ml of mineral salts solution was found to be the maximum amount if filtering was to be completed in a reasonable time, this quantity was used in the flasks on the shaker. Four replicates were used for each variable. The shaker was stopped for a 4-hour period each day, and each flask was swirled gently to remove any ring which may have formed on the sides of the flask. Incubation was at 30 C, though temperatures sometimes

went slightly above that. At the completion of each experiment, the residue was treated as described above.

The effect of flask size on the rate of cellulosic decomposition by the two organisms was first studied.

With *S. myxococcoides*, shaking increased the rate of decomposition four-fold, whereas with *Cellulomonas* sp. the rate was merely doubled. The former is, therefore, a much more aerobic organism than the latter. The odd position which the *Cellulomonas* sp. occupies is made clear from table 3. As the depth of the suspension increases, the rate of decomposition increases. But sealing lowers the rate, the inhibition increasing with decreasing air volume of the flask. It appears that abundant air retards decomposition by this organism. Erlenmeyer flasks of 125- or 250-ml volume are adequate for *S. myxococcoides*, but the 50-ml size is superior for decomposition by *Cellulomonas* sp.

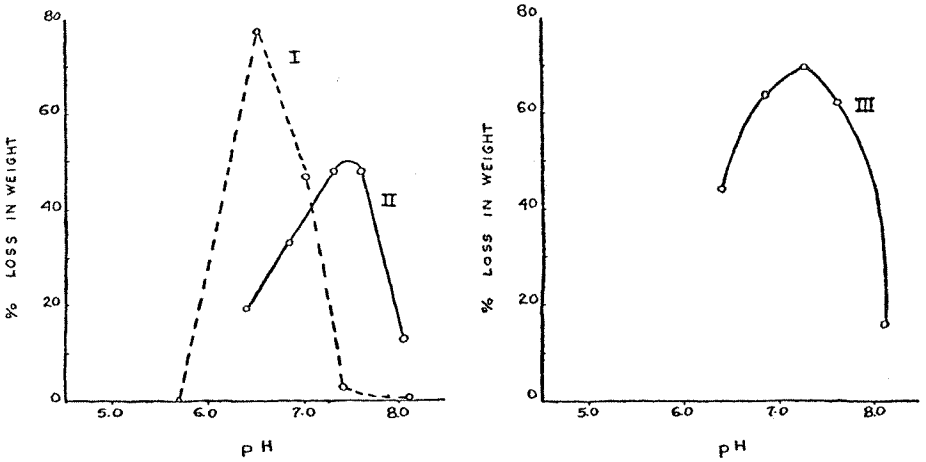

FIG. 2. EFFECT OF pH ON DECOMPOSITION OF FILTER PAPER IN SHAKE FLASKS BY MICROORGANISMS

Curve I, *S. myxococcoides* (4 days); curve II, *Cellulomonas* sp. (6 days); curve III, *Actinomyces* sp. (PQD-B36D) (6 days).

EFFECT OF HYDROGEN ION CONCENTRATION

The effect of pH on decomposition may be related to the nature of the medium. In the present experiments, the medium for *S. myxococcoides* was a mineral salts solution containing 12 ppm iron and no organic matter but the cellulose. The medium for the other organisms contained, besides the salts, urea as a source of nitrogen, yeast extract, and gelatin. In all cases, the optima for cellulose decomposition fall within the pH range 6.5 to 7.5.

EFFECT OF NITROGEN SOURCE

In studying the effect of the nitrogen source on the rate of decomposition, two nitrates, two ammonium compounds, and urea were selected (table 4). The nitrogen source was added in quantity equivalent to 0.165 g nitrogen per liter.

For both organisms ammonium nitrogen is as good as nitrate nitrogen, but all ammonium compounds are not of equal value. The effect of the anion is quite definite, the carbonate being superior to the sulfate, though the difference in pH may be the deciding factor. As the ammonium nitrogen is utilized, the

TABLE 4

Effect of nitrogen source on rate of decomposition of cellulose by S. myxococcoides and Cellulomonas sp.

NO.	NITROGEN SOURCE	*S. myxococcoides* 4 days			*Cellulomonas* sp. 6 days		
		pH		Avg % loss*	pH		Avg % loss* in weight
		Orig.	Final		Orig.	Final	
A	$NaNO_3$	6.8	7.7	66	7.3	8.0	46
B	$Mg(NO_3)_2$	6.4	7.7	65	6.8	7.5	48
C	$(NH_4)_2CO_3$	6.9	5.5	61	7.7	5.0	43
D	$(NH_4)_2SO_4$	6.5	6.2	25	6.8	4.7	27
E	Urea	6.9	7.2	3	7.3	6.7	68

* Average of 4 replicates.

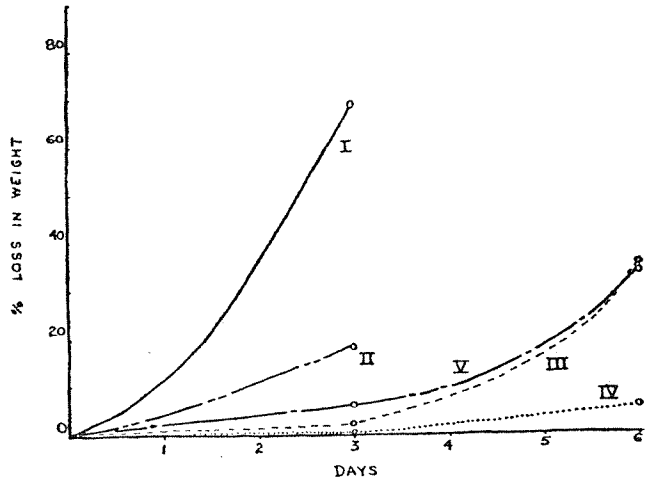

FIG. 3. EFFECT OF UREA CONCENTRATION ON RATE OF DECOMPOSITION BY S. MYXOCOCCOIDES (IN THE PRESENCE OF NITRATE N)

I = no urea; II = 0.05 g N per liter as urea; III = 0.14 g N per liter as urea; IV = 0.41 g N per liter as urea; V = IV plus *E. coli* inoculum.

pH drifts, the drift to the acid side being much greater for *Cellulomonas* sp. (pH 4.7) than for the *S. myxococcoides* (pH 6.2). It may be that urea is a good nitrogen source for *Cellulomonas* sp. because there is so little change in pH during its utilization.

Urea exerts an interesting influence on these organisms. In the case of *S. myxococcoides*, not only is it not available (table 4), but it is actually toxic

(figure 3). When it is added to the usual medium containing $NaNO_3$, it considerably reduces the rate of decomposition. Winogradsky (1939) reported a similar inhibition by 0.3 M urea in the case of another organism, *Nitrobacter*. Our results show a much higher degree of toxicity, 0.002 M urea slowing down the rate significantly. A light *Escherichia coli* inoculum added to the solution of highest urea concentration increases the rate of decomposition over that shown without the *E. coli*, apparently by reducing the urea concentration from 0.41 to approximately 0.14 g N per liter. (This high degree of toxicity of urea does not apply to its use in agar, where fabric strips are being decomposed on the agar surface.)

In direct contrast with the foregoing is the effect of urea on *Cellulomonas* sp. (table 4). This bacterium prefers urea nitrogen. The third possibility,

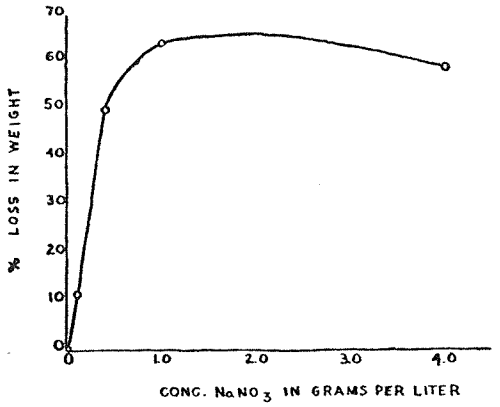

FIG. 4. EFFECT OF $NaNO_3$ CONCENTRATION ON RATE OF DECOMPOSITION OF FILTER PAPER BY S. MYXOCOCCOIDES (3 DAYS' INCUBATION)

urea nitrogen being equal to nitrate nitrogen, is exemplified by *Actinomyces* sp. (PQD-B36D). At the end of 6 days' incubation on the shaker, the medium containing urea gave 59 per cent loss in weight while that containing sodium nitrate also gave 59 per cent loss.

EFFECT OF OTHER SALTS

Examination of the value of other salts in the medium indicated that for both bacteria potassium chloride had practically no effect up to a concentration of 2.5 g per liter. Above this concentration, a toxic action is apparent (figure 5), which is probably a result of the high total salt concentration. When studying the action of a particular salt, therefore, it would appear to be necessary to vary the concentration of the other salts, so that the total molar concentration remains constant. This is especially important when the concentration being studied exceeds 0.06 N. Above that point, the effects of the variable would be obscured by the high total concentration.

Magnesium sulfate is important to both bacteria. The addition of 0.5 g per liter of this salt nearly doubled the rate of decomposition. Iron salts are stimu-

latory to *S. myxococcoides* but show no such effect on *Cellulomonas* sp. (figure 6). If, however, the phosphate concentration is increased from 0.01 M to 0.06 M, the stimulatory effect of iron on *S. myxococcoides* disappears.

Results with other minor elements (Cu, Zn, B, Mo, Mn) show no stimulation whatsoever. Copper is toxic[2] to both organisms at 1 ppm. Manganese is

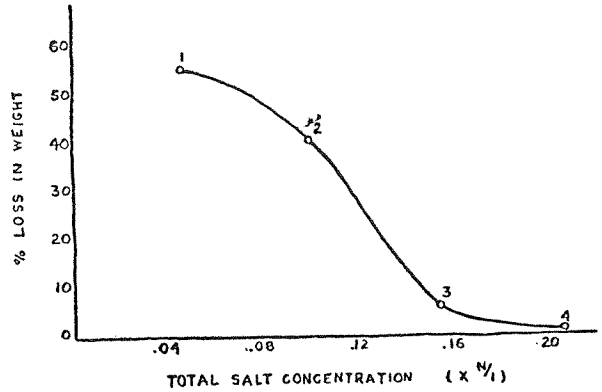

FIG. 5. EFFECT OF INCREASING POTASSIUM CHLORIDE CONCENTRATION ON DECOMPOSITION OF FILTER PAPER BY S. MYXOCOCCOIDES (3 DAYS)

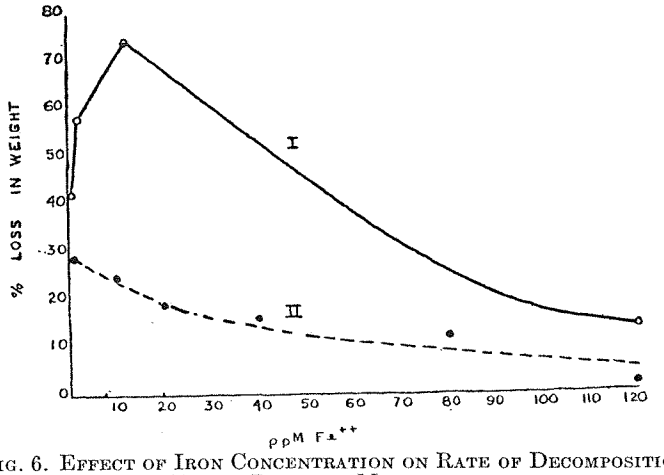

FIG. 6. EFFECT OF IRON CONCENTRATION ON RATE OF DECOMPOSITION OF FILTER PAPER BY MICROORGANISMS
Curve I, *S. myxococcoides* (5 days); curve II, *Cellulomonas* sp. (6 days).

toxic to *Cellulomonas* sp. at 1 ppm but is without effect on *S. myxococcoides*, even at 5 ppm.

EFFECT OF YEAST EXTRACT AND GELATIN

In the aeration experiments, there was an indication that yeast extract and gelatin might be of some value to *S. myxococcoides* in aerated flasks. Since

[2] By toxic here is meant a decrease in the rate of decomposition from about 50 per cent for controls to less than 5 per cent for flasks containing the element in question.

only 12 per cent decomposition was obtained in 6 days, however, shaker tests (table 5) were run to clarify the issue. The results indicate that no advantage is gained by the addition of these substances to the medium found optimum for this organism. On the other hand, results with *Cellulomonas* sp. on the shaker (table 6) confirm those of the aeration tests regarding the necessity of yeast and of gelatin for maximum decomposition.

TABLE 5

Effect of yeast extract and gelatin on cellulose decomposition by S. myxococcoides in shaker flasks

NO.	VARIATION		MG RESIDUE					AVG % LOSS IN WEIGHT	pH	
	% gelatin	% yeast extract	1	2	3	4	Avg		Orig.	Final
1	0	0	24	27	29	33	28	71	7.2	7.9
2	0	0.004	26	27	27	30	28	71	7.2	7.9
3	0	0.04	31	32	37	—	33	66	7.2	7.9
4	0.004	0.004	26	29	30	30	29	70	7.2	7.9
5	0	0	24	29	31	—	28	71	7.2	7.9
6	Uninoc. controls		94	96	96	99 97	94.4	—	—	—

TABLE 6

Effect of yeast extract and gelatin on decomposition of filter paper by Cellulomonas sp. (6 days on shaker)

NO.	VARIATION		MG RESIDUE					AVG % LOSS IN WEIGHT	pH	
	% gelatin	% yeast extract	1	2	3	4	Avg		Orig.	Final
1	0	0	102	102	99		101	0	7.5	7.2
2	0	0.004	75	75	78		76	25	—	7.4 7.5
3	0.004	0.004	71	74	67		71	30	7.5	7.5 7.7
4	0.004	0.04	35*	37	35		36	64	7.5	6.5
5	0.04	0.004	79	74	72		75	26	7.5	6.3 6.4
6	Uninoc. checks		101	102	99	100	101	—	—	—

* Five-day result.

DECOMPOSITION RATE AT OPTIMAL CONDITIONS

Under conditions believed to be optimal for each organism, the rate of decomposition of cellulose in shaker flasks was determined. For *S. myxococcoides* the medium contained M/1 potasium phosphate buffer (pH 6.7), 10 ml; $NaNO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.05 g; and the cellulose at 4 g per liter. For *Cellulomonas* sp., the medium consisted of urea, 0.356 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; M/1 potassium phosphate buffer (pH 7.6), 10 ml; yeast extract, 0.3 g; gelatin, 0.03 g; and ground filter paper, 4 g per liter. The first medium was placed in 250-ml flasks, and the second in 50-ml flasks. The cultures were removed from the shaker at various intervals, and the loss in cellulose was determined. The results of this experiment are plotted in figure 7.

Cellulomonas sp. decomposed filter paper at a rate equal to that of *S. myxococcoides*. The figure, 50 per cent loss in weight in 3 days, is, however, not equal to the best obtained by the latter. It is the highest rate obtained by the *Cellulomonas* sp. in any of the present experiments.

All curves finally level off while the undissolved residue remaining is still over 20 per cent. Just how much of the residue is bacterial substance and how much cellulose has not been determined.

With *Cellulomonas* sp., there was a continued drop in pH. Thus, the final pH values in either case were near the extreme end of the range of activity for each organism, so that any remaining cellulose would be decomposed at a very slow rate. Calcium carbonate added to three flasks of *Cellulomonas* sp. at the

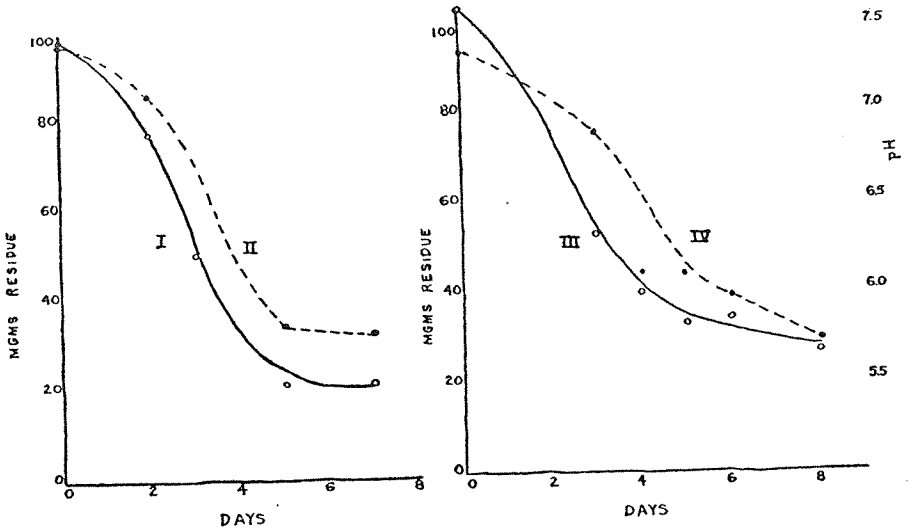

FIG. 7. RATE OF DECOMPOSITION OF CELLULOSE

S. myxococcoides, curve I, filter paper; curve II, cotton cloth.
Cellulomonas sp., curve III, filter paper; curve IV, pH vs. time.

beginning of the experiment kept the pH up to 6.8 to 7.0 at the end of 5 days (compared to 6.1 without calcium carbonate), but the amount of final residue (36 mg) was not significantly different from that without calcium carbonate (33 mg). It appears, therefore, that the residue is mainly bacterial substance and not cellulose.

It is believed that the two organisms studied, isolated from fabric samples, should prove useful in tests designed to determine the resistance of treated cotton fabric to microbial action. It is of the utmost importance that organisms showing the greatest *dissimilarity* be used in such tests. *S. myxococcoides* and the fungi are alike in many respects. They are highly aerobic and show a preference for low pH, but they differ in mode of action on the fiber. *Cellulomonas* sp. differs from all in being most active as anaerobic conditions are approached.

APPLICABILITY OF SHAKER METHOD TO FUNGI

To this point, data have been presented relative to the use of aeration and particularly of shaker flasks, in a study of cellulose-decomposing bacteria. The applicability to fungus studies was next investigated. Five fungi of different cellulose-decomposing ability were selected. *Aspergillus niger* (J745) was included because of conflicting reports on its ability to utilize cellulose. The other fungi are recognized cellulose destroyers.

Humicola, *Metarrhizum*, and *Chaetomium* formed heavy rings of deposited material on the flask above the liquid level. These were not washed down during the course of the experiment. At the end of the incubation period, the residues were treated with 0.1 volume M/1 potassium hydroxide and autoclaved, as was

TABLE 7

Decomposition of filter paper in shaker flasks by various fungi

FUNGUS	DAYS INCUBATED	MG RESIDUE			AVG % LOSS IN WEIGHT
		1	2	Avg	
Chaetomium globosum, USDA 1042.4	3	90	89	90	14
Humicola sp., PQMD 34e	3	50	56	53	50
Metarrhizum glutinosum, USDA 1334.2	3	59	64	62	41
Uninoc. controls	3	103	105		—
		105	107	105	
Aspergillus terreus, MIT 7	6	52	49	51	51
Aspergillus niger, J745	6	104	106	105	0

customary with the bacterial residues. In spite of the ring formation, the results of duplicate flasks were in good agreement, and the rate of breakdown was rapid. Furthermore, the descending order of activities (*Humicola* and *Metarrhizum*, *Chaetomium*, *Aspergillus terreus*, *A. niger*) is the same as that determined for these organisms by Dr. W. L. White, of the Tropical Deterioration Research Laboratory, using the loss in tensile strength of fabric on agar as a criterion. *A. terreus*, which was cultured for 6 days, formed less of a ring, and the ring was washed down daily. This would tend to result in more uniform action than would be found with the heavy rings of the faster-growing fungi.

SUMMARY

Two methods for studying cellulose decomposition quantitatively were investigated. Both methods gave good results. Aeration involved the problem of preventing contamination and wetting plugs by bubble formation. The use of shaker flasks at 120 cycles per minute was shown to be an excellent method for the study of microbial decomposition of cellulose.

The optimal conditions for two cellulose-decomposing organisms were partially worked out. It was shown that *S. myxococcoides* is a strongly aerobic organism,

capable of high rates of decomposition of pure cellulose. It is greatly stimulated by 10 ppm Fe^{++} and has its optimal pH near 6.5. No growth factors appear to be necessary. *Cellulomonas* sp. requires very little oxygen for its optimal rate of decomposition but is dependent upon an external source of growth substances. It is not stimulated by iron, has an optimal pH near 7.5, and utilizes urea nitrogen preferentially to nitrate nitrogen.

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